Supplementary Online Material

1. Materials and Methods

Gene targeting construct

The gene targeting construct was made using a 7.2kb genomic fragment including exons 3 and 4 of *Mecp2* (*1*). The plasmid was linearised by partial digestion with NcoI and blunted using Klenow enzyme. A floxed STOP-Neo cassette (2) was excised from its vector as a 3.1kb XbaI/Asp718I fragment, blunted, and inserted into the blunted NcoI site in intron 2 of *Mecp2*. The resulting targeting vector was linearised at the 5' end using SalI.

ES cell culture and gene targeting

Gene targeting was performed as described (*1*) with E14TG2a ES cells (derived from 129 Ola mice). Correctly targeted clones were identified by Southern blotting of genomic DNA digested with HindIII and NdeI and probed with a 1.1kb HindIII-BamHI fragment from the 3'UTR of *Mecp2*.

Generation and breeding of chimeric mice

Correctly targeted ES cells were injected into C57Bl6 blastocysts and transferred to pseudopregnant recipient females by standard methods. Chimeric pups were identified by their agouti coat colour and were test mated to C57Bl6 mice. As *Mecp2* is X-linked, all resulting agouti females were heterozygous for the *lox-Stop* allele. The line was maintained by breeding heterozygous females to C57Bl6 males and genotyped by PCR on genomic DNA prepared from identifying ear biopsies. The wt allele was identified using primers p5 (TGGTAAAGACCCATGTGACCCAAG) and p7 (GGCTTGCCACATGACAAGAC) resulting in a band of 416bp, and the *lox-Stop* allele using primers NeoF (GTCATCTCACCTTGCTCCTGCC) and NeoR (GAAGGCGATAGAAGGCGATGCG) giving a 470bp band.

Mice were maintained under standard conditions and in accordance with Home Office regulations and licences. Mice were carefully monitored for symptoms due to either the genetic mutation or the experimental treatment, and animals that exceeded the severity limit for this experiment were humanely culled.

Scoring of symptoms

Mice were scored for a number of symptoms arising from MeCP2 deficiency. These tests were suitable for weekly assessment over a prolonged period to generate a semi-quantitative measure of symptom status. Observations were made whilst handling the mice at a laboratory bench, always at the same location and, where possible, at the same time of day. Where available, mice were compared with wild-type littermates. Each of 6 symptoms was scored as 0 (absent or as wild-type), 1 (symptom present) or 2 (symptom severe). Mice were also weighed at each scoring session.

1. **Mobility:** The mouse is observed when placed on bench, then when handled gently. 0 = as wild-type. 1 = reduced movement when compared to wild-type: extended freezing period when first placed on bench and longer periods spent immobile. 2 = no spontaneous movement when placed on the bench; mouse can move in response to a gentle prod or a food pellet placed nearby. (Note: mice may become more active when in their own cage environment.)
2. **Gait:** 0 = as wild-type. 1 = hind legs are spread wider than wild-type when walking or running with reduced pelvic elevation, resulting in a “waddling” gait. 2 = more severe abnormalities: tremor when feet are lifted, walks backwards or 'bunny hops' by lifting both rear feet at once.
3. **Hindlimb clasping:** Mouse observed when suspended by holding base of the tail. 0 = legs splayed outwards. 1 = hindlimbs are drawn towards each other (without touching) or one leg is drawn in to the body. 2 = both legs are pulled in tightly, either touching each other or touching the body.
4. **Tremor:** Mouse observed while standing on the flat palm of the hand. 0 = no tremor. 1 = intermittent mild tremor. 2* = continuous tremor or intermittent violent tremor
5. **Breathing:** Movement of flanks observed while animal is standing still**.** 0 = normal breathing. 1 = periods of regular breathing interspersed with short periods of more rapid breathing or with pauses in breathing. 2* = very irregular breathing - gasping or panting.
6. **General condition:** Mouse observed for indicators of general well-being such as coat condition, eyes, body stance. 0 = clean shiny coat, clear eyes, normal stance. 1 = eyes dull, coat dull/ungroomed, somewhat hunched stance. 2* = eyes crusted or narrowed, piloerection, hunched posture.

Each category was scored and the digits totalled to give an aggregate number that could be displayed graphically (e.g., 112010 = 5) and used to express overall phenotype.

The score was used to determine when animals should be culled for humane reasons. An animal scoring 2 for any of the categories marked * above would routinely be culled, as would any animal showing a reduction of 20% of its pre-symptomatic body weight (apart from obese animals). Thus animals referred to in the text (for reasons of brevity) as having "died" were invariably culled according to the above criteria.

Reactivation of the *Mecp2* gene

Mice carrying a CreESRT (*cre-ER*) transgene (3) were obtained from the Jackson Laboratory (strain name:B6.Cg-Tg(cre/ESR1)5Amc/J, stock number 004682) and maintained by crossing hemizygotes. The transgene was identified by PCR using primers CreF (GACCGTACACCAAAATTTGCCTGC) and CreR (TTACGTATATCCTGGCAGCGATC) to give a 465 bp product.

Heterozygous *Stop/+* females were crossed with males hemizygous for the *cre-ER* transgene to produce mice with both the *cre-ER* transgene and the *Mecp2 lox-Stop* allele, which were used in experiments. Wild-type and *lox-Stop*-only littermates were used as controls in deletion experiments. Mice were treated with tamoxifen (TM) (Sigma) in order to reactivate *Mecp2* by Cre-mediated excision of the *lox-Stop* cassette. Tamoxifen was dissolved in Corn Oil (Sigma) at 20mg/ml by brief sonication and then stored at -20ºC until required. Mice were treated by intra-peritoneal injection at 100mg/kg body weight for each treatment. Initially mice were treated daily for five consecutive days but, when the toxicity due to reactivation of MeCP2 became apparent, the treatment was changed to one injection per week for five weeks followed by a booster of three daily injections after a variable period of time (see Figs 2 and 3).

To measure recombination of the *Mecp2 lox-Stop* allele, genomic DNA was prepared from mouse brains. DNA was digested with EcoRI and NcoI and Southern blots were probed with a 1.1kb HindIII fragment covering most of the ORF contained in exon 4 of *Mecp2* (see Fig. S1). Recombination was quantified with a Storm PhosphorImager and ImageQuant software.

Western-blot analysis and immunofluorescence

Protein extracts for western blot analysis were prepared from whole brains. Brains were collected into ice cold phosphate buffered saline (PBS) and briefly homogenized in a Dounce homogeniser with the loose pestle. Cells were passed through a 40µm Cell Sieve (BD Biosciences), washed in PBS, resuspended in Lysis buffer (25mM HEPES pH7.8, 25mM KCl, 5mM $MgCl_2$, 0.05mM EDTA, 10% glycerol, 0.1% NP40, 0.1mM PMSF, 1mM DTT) and left on ice for 5 minutes. Nuclei were washed in lysis buffer and then resuspended in SDS-PAGE sample buffer (50mM Tris.HCl pH6.8, 2% SDS, 10% glycerol, 10mM DTT), boiled for 5 minutes and briefly sonicated to shear genomic DNA. Samples were centrifuged for 5 minutes at 13,000rpm and the supernatants stored at -80ºC. Western blotting was performed as described (*1*) with a commercial anti-MeCP2 antibody (Upstate; 1:2000 dilution) or an antibody raised against the C-terminus of MeCP2 (*4*) (1:1000 dilution) or a histone H4 antibody (Upstate; 1:2000 dilution) as a loading control.

Immunofluorescence was performed on cryosections of mouse brain. Brains were dissected from freshly sacrificed animals, the rostral and caudal parts (including olfactory bulb and cerebellum) were removed and the remainder cut in half sagittally and snap frozen in liquid nitrogen. Sections were cut at 5µm thickness using a cryostat (Leica) and thaw-mounted onto Superfrost Plus Gold microscope slides (Menzel-Glaser). Sections were fixed in 3.7% paraformaldehde in CSK buffer (100mM NaCl, 300mM sucrose, 10mM PIPES pH6.8, 3mM $MgCl_2$, 1mM EDTA) for 30 minutes at room temperature (RT), washed (all washes in PBS, twice 10 minutes at RT) and permeabilized in 0.1% Triton X-100 in CSK buffer, 15 minutes at RT. Sections were blocked overnight at 4ºC with 1.5% normal goat serum in PBS (= BB) in a humidified chamber, then incubated with anti-MeCP2 antibodies either from J. Pevsner (*4*) or from Upstate in BB for 2 hours at RT. After washing, sections were incubated with biotinylated goat anti-rabbit igG (Vectorlabs) diluted 1:200 in BB for 1 hour at RT, washed, and incubated with fluoroscein avidin DCS reagent (Vectorlabs) 1:200 in HEPES-buffered saline (15 min). Sections were washed, mounted in Vectashield+DAPI (Vectorlabs) and images made using a Zeiss Axioskop 2 fluorescence microscope (20x objective lens).

Electrophysiology

Mice were killed by cervical dislocation and brains transferred to ice-cold oxygenated (95% O_2, 5% CO_2) artificial cerebrospinal fluid (in mM): NaCl, 124; KCl, 3; $NaHCO_3$, 26; NaH_2PO_4, 1.25; $MgSO_4$, 1; glucose, 10; $CaCl_2$, 2. Transverse hippocampal slices

(400 µm thick) were prepared and transferred to an interface-type tissue chamber maintained at 32 °C, as described (*5*). Standard extracellular recording was used to monitor field excitatory postsynaptic potentials (fEPSPs) in stratum radiatum of area CA1 in response to Schaffer-collateral afferent stimulation (range = 0-30 mA). The slope of the evoked fEPSPs was measured and expressed relative to the pre-conditioning baseline. Responses were set to approximately 50% of the maximal response (9.2 ± 0.7 mA stimulation strength which did not differ significantly between LTP groups). fEPSPs were evoked at a frequency of 0.05 Hz and LTP induced by either high frequency stimulation (single train of 100 stimuli at 100 Hz) or theta burst stimulation (10 trains of 4 pulses at 100 Hz with an inter-train interval of 0.2 s). Recordings were made with an Axoclamp 2B amplifier (Molecular Devices, Sunnayvale, CA, USA), monitored and analysed on-line and re-analyzed off-line (*6*). Data pooled across slices are expressed as the mean ± SEM and differences between LTP groups calculated on the average slope over a 40-60 minute period after the conditioning stimuli.

2. Captions to Supplementary Movies

Movie S1

The movie shows a *Mecp2*$^{lox\text{-}Stop/y}$,*cre-ER* mouse (#286, see Figure 2D) with neurological symptoms at 12 weeks of age. Note low stance, inertia, tremor, arrhythmic breathing, splayed hind limb position and moderate hindlimb clasping. TM injection was initiated on this day.

Movie S2

The same mouse as shown in Supp.Movie_1 four weeks later after a course of 5 weekly TM injections.

Movie S3

Female mice that received identical TM administration regimes 26 weeks prior to filming. The first mouse seen is a *Stop/+,cre* female that displayed symptoms at the beginning of TM treatment and is now indistinguishable from wildtype using our symptom scoring system (see Supplementary Methods). The second mouse entering the frame is a wildtype female. The third mouse to appear is a *Stop/+* female lacking the cre-ER transgene, which therefore fails to respond to TM. Note inertia and obesity of this third mouse.